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(71) Applicant: IBEX TECHNOLOGIES R AND D, INC. [CA/CA]; 5485 Pare, Montreal, Quebec H4P 1P7 (CA).

(71)(72) Applicant and Inventor: ZIMMERMANN, Joseph [US/US]; 13450 Nicolet Avenue, Elm Grove, WI 53122 (US).

(72) Inventors: BENNETT, D., Clark; 4965 Hortie, Pierrefonds, Quebec H4Y 1Z4 (CA). LALIBERTE, Maryse; 252 Avenue Colombier, Boisbriand, Quebec J7G 1L2 (CA). GU, Kangfu; 106 Viking Place, Dollard des Ormeaux, Quebec H9G 2P1 (CA). TKALEC, Anna, Lydia; 6250 Langelier, Montreal, Quebec H1M 2B8 (CA). FINK, Dominique; 4548 Chambord, Montreal, Quebec (CA). LINHARDT, Robert; 1422 Plum Street, Iowa City, IA 52240 (US).

(74) Agent: PABST, Patrea, L.; Arnall Golden & Gregory, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, GA 30309-3450 (US).

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(57) Abstract

The present invention describes a method for the production of two highly purified enzymes capable of degrading chondroitin sulfate polysaccharides. A multi-step purification method incorporating cell disruption, cation exchange chromatography, affinity chromatography, hydroxylapatite chromatography, high resolution ion exchange chromatography and size exclusion is outlined. A 77,000 ± 5,000 Dalton protein capable of degrading chondroitin sulfates A and C and a 55,000 ± 2,300 Dalton protein capable of degrading dermatan sulfate were isolated. The genes encoding these enzymes, chondroitinase AC and chondroitinase B, respectively, have been cloned and methods for their use are described.

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CHONDROITIN LYASE ENZYMES

Background of the Invention

The present invention is the purification and cloning of chondroitin lyase enzymes found in *Flavobacterium heparinum.*

Glycosaminoglycans are unbranched polysaccharides consisting of alternating hexosamine and hexuronic residues which carry sulfate groups in different positions. This class of molecules can be divided into three families according to the composition of the disaccharide backbone. These are: heparin/heparan sulfate [HexA-GlcNAc(SO_4)]; chondroitin sulfate [HexA-GalNAc]; and keratan sulfate [Gal-GlcNAc]. The chondroitin sulfate family includes seven sub-types designated unsulfated chondroitin sulfate, oversulfated chondroitin sulfate and chondroitin sulfates A-E which vary in the number and position of their sulfate functional groups. Additionally, chondroitin sulfate B, also referred to as dermatan sulfate, differs in that iduronic acid is the predominant residue in the alternative hexuronic acid position.

Chondroitin sulfates A, B and C are the predominant forms found in mammals and may be involved in the modulation of various biological activities including cell differentiation, adhesion, enzymatic pathways and hormone interactions. The presence of chondroitin sulfate proteoglycans is elevated in the later stages of cell growth in response to tissue and vessel damage, as reported by Yeo, et al., *Am. J. Pathol.* 138:1437-1450, 1991, Richardson and Hatton, *Exp. Mol. Pathol.* 58:77-95, 1993 and Forrester, et al., *J. Am. Coll. Cardiol.* 17:758-769, 1991. Chondroitin sulfates also have been associated with events involved in the progression of vascular disease and lipoprotein uptake as described by

Tabas, et al., *J. Biol. Chem.*, 268(27):20419-20432, 1993.

Chondroitin enzymes of a suitable purity and characterization could be useful tools in determining the role of chondroitin sulfates in modulating these cellular events and in developing therapeutics for the treatment of disease states.

Chondroitin sulfate degrading enzymes, referred to as chondroitinases or chondroitin sulfate lyases, from several bacterial species have been reported. Takegawa, et al., *J. Ferm. Bioeng.* 77(2):128-131, 1991, report a chondroitinase AC from *Aureobacterium* with a molecular weight of between 81,000 and 83,000 Daltons that is inhibited by copper ions. *Bacteriodes thetaiotamicron* produces two chondroitinase AC degrading enzymes of molecular weight 104,000 and 108,000 Daltons, as described by Linn, et al., *J. Bacteriol.* 165:859-866, 1985. Other bacterium including *Flavobacterium heparinum, Proteus vulgaris, Arthrobacter aurescens* and *Pseudomonas fluorescens* produce chondroitinase AC or chondroitinase ABC enzymes which are not well characterized, as reviewed by Linhardt, et al., *Appl. Biochem. Biotechnol.* 12:135-177, 1986. *F. heparinum* is the only microbe that produces an enzyme which is specific for dermatan sulfate, chondroitinase B, as reported by Linhardt, R., et al. However, the chondroitinase degrading enzymes from *F. heparinum* have not been purified to homogeneity or thoroughly characterized.

It is therefore an object of the present invention to provide methods for purifying chondroitin lyase enzymes.

It is a further object of the present invention to provide DNA sequences encoding chondroitin lyase enzymes.

It is a still further object of the present invention to provide purified chondroitin lyase enzymes which are useful as pharmaceutical regents.

Summary of the Invention

A method for purifying chondroitin lyase enzymes from bacteria such as the Gram negative organism, *Flavobacterium heparinum*, have been developed which yields purified chondroitinase AC and chondroitinase B. Cells are grown by fermentation culture, the cells are lysed preferably using an osmotic shock technique which selectively releases proteins from the periplasmic space, then fractionated by cation exchange chromatography. Fractions containing chondroitinase degrading activity are further fractionated by affinity chromatography using a sulfated cellulose based resin and hydroxylapatite chromatography which separate the chondroitinase AC and chondroitinase B activities. Highly purified preparations of each enzyme are obtained by an additional chromatography step using a high resolution strong cation exchange resin. Pure preparations of chondroitinase B may require an additional separation step based on molecular size, such as gel filtration liquid chromatography.

The genes encoding chondroitinase AC and chondroitinase B enzymes of *Flavobacterial* origin were cloned. These can be used in conjunction with suitable expression systems to produce the enzymes in *Flavobacterium*, for example, under the control of overexpression promoters, or in organisms other than *Flavobacterium*.

Brief Description of the Drawings

Figure 1 is a schematic of the construction of plasmids used to sequence the chondroitinase AC

gene from *Flavobacterium heparinum*, pA2C1B, p64BS2-7. Restriction sites are: S - *SaU*, B - *Bam*HI, P - *Pst*I, E - *Eco*RI, H - *Hind*III, C - *Cla*I and K - *Kpn*I.

Figure 2 is a schematic of the construction of pGB-ChAC, a plasmid capable of directing the expression of active chondroitinase AC in *E. coli* from tandem tac promoters (double arrowheads).

Figure 3 is a schematic of the construction of plasmids used to sequence the chondroitinase B gene from *Flavobacterium heparinum*, pCHB300 and pCHB78.

Figure 4 is a schematic of the construction of pGB-CHB, a plasmid capable of directing the expression of active chondroitinase B in *E. coli* from tandem tac promoters (double arrowheads).

Detailed Description of the Invention

Purification of Chondroitin Sulfate degrading Enzymes from *F. heparinum*

Cells are grown in fermentation cultures to obtain sufficient quantities of the enzymes. Chondroitin sulfate A is included in the media at a concentration of between 0.5 and 10 g/l, preferably between 1.0 g/L to 2.0 g/l to induce chondroitinase AC and chondroitinase B synthesis. Crude enzyme extracts are prepared by liberating soluble proteins from the cells by standard cell disruption techniques, preferably osmotic shock based techniques which selectively release proteins from the cell's periplasmic space. For example, proteins can be released from the periplasmic space by treatment with non-ionic detergents in the range of 0.01 to 1.0%, freezing and thawing the cells, partial sonication for 0.5 to 6.0 minutes at 30 to 60% power in a pulsed mode 25/75 to 75/25, lysosyme

treatment at 0.001 to 1.0 mg/ml for 15 to 60 minutes between 4 and 25°C, organic solvent treatment with 0.01 to 1.0% chloroform or toluene or by the osmotic shock process described in U.S. Patent No. 5,169,772 to Zimmermann and Cooney. In the latter, cells are partially sonicated for between 0.5 and 4.0 minutes, poser 3-6 pulsed mode 50/50, partial homogenization 250 to 500 psi, followed by lysozyme treatment at 0.001 to 1.0 mg/ml for between 15 and 60 minutes at between 4 and 23°C, and organic solvent treatment with 0.01 to 1.0% chloroform or 0.01 to 1.0% toluene.

In the preferred embodiment, the crude extract is fractionated by cation exchange chromatography using a high flow rate resin such as Sepharose™ S Big Beads (Pharmacia), MonoS™ (Pharmacia), CBX (J.T. Baker), Sepharose™ S (Pharmacia), and CM cellulose (Bio-Rad or Sigma), at a pH of between 6.0 and 8.5 with a salt gradient equivalent to 0.01 to 1.0 M NaCl. The bound proteins are preferably eluted with step gradients of 0.25 M sodium chloride and 1.0 M sodium chloride, at pH 7.0. Chondroitinase activity elutes in the 0.25 M sodium chloride fraction. Other salts can be utilized, such as sodium phosphate or sodium sulfate to create the salt gradient. Alternatively, a pH gradient in the range of 6.0 to 10.0 could be employed or a combination of a salt and pH gradient.

Fractions containing chondroitinase degrading activity are further fractionated by affinity chromatography using a sulfated cellulose based resin with a linear gradient of 0.0 to 0.4 M NaCl. Chondroitinase AC primarily elutes at 0.23 to 0.26 M NaCl and chondroitinase B elutes at 0.27 to 0.3 M NaCl. This is followed by hydroxylapatite chromatography using a step gradient of 0.25 M NaCl

followed by a linear gradient of 0.25 to 1.0 M NaCl at pH 7.7. Chondroitinase B elutes at 0.25 M NaCl while chondroitinase AC elutes at 0.85 to 0.95 M NaCl. Highly purified preparations of each enzyme are obtained using a high resolution strong cation exchange resin eluted with a linear gradient from 0.125 to 0.325 M NaCl in 0.025 M sodium phosphate at pH 7.0 ± 0.1, as described with reference to elution from cation exchange resins described above. Chondroitinase B elutes in a protein peak at 0.175 to 0.225 M NaCl. Chondroitinase B can be further purified on the basis of molecular size by size exclusion chromatography, ultrafiltration or preparative gel electrophoresis. Gel filtration (size exclusion) resins with maximum resolution performance in the range of 5,000 to 100,000 are preferred. These include Superose™ 12, Superose™ 6, Sephadex™ G-50 and Sephadex™ G-50 from Pharmacia and BioGel™ P-60 and BioGel™ P-100 from BioRad. Ultrafiltration or dialysis membranes with molecular weight cutoffs in the range of 10,000 to 30 000 Daltons are useful in removing small contaminants while ultrafiltration and dialysis membranes with molecular weight cut-offs in the range of 70,000 to 1,000,000 Daltons are useful to remove larger contaminants. Alternatively, chondroitinase B containing samples of sufficient purity, more than 25% pure, could be further purified by subjecting the sample to gel electrophoresis according to standard laboratory procedures, and excising the major band appearing at a molecular weight of 55,000 ± 2,300 Daltons.

The method of producing and purifying the chondroitinase lyase enzymes is exemplified as follows.

F. heparinum was cultured in 15 L computer controlled fermenters in a variation of the defined

nutrient medium described by Galliher, et al., *Appl. Environ. Microbiol.* 41(2):360-365, 1981. Chondroitin sulfate A (Sigma) was included in the media at a concentration of 1.0 g/L as the inducer of chondroitinase AC and chondroitinase B synthesis. The cells were harvested by centrifugation and the desired enzymes released from the periplasmic space by a variation of the osmotic shock procedure described by U.S. Patent No. 5, 169,772 to Zimmermann and Cooney. Cells were resuspended in 0.01 M sodium phosphate and 0.3 M sodium chloride at pH 7.0 ± 0.1 to give a final cell concentration of 100 absorbance units at 600 nm. The non-ionic detergent Nonedit™ P-40 was added to the cell suspension to a final concentration of 0.1% and the cells stirred for 1 hour at room temperature using a magnetic stir bar device. Cells and cell debris were then removed by centrifugation using a Sorval™ RC5C centrifuge with a JA-10 rotor at 10,000 RPM for 45 minutes. The cell pellet was discarded and the osmolate supernatant retained for further processing.

Osmolates obtained from *F. heparinum* fermentations induced with chondroitin sulfate A were subjected to centrifugation to remove cells and cell debris and the supernatant applied to a cation exchange column (5.0 cm x 30 cm, Sepharose™ S Big Beads, Pharmacia) at a linear flow rate of 10 cm•min^{-1}. The bound proteins were eluted at a linear flow rate of 5.1 cm•min^{-1} with step gradients of 0.01 M phosphate, 0.01 M phosphate/0.25 M sodium chloride and 0.01 M phosphate/1.0 M sodium chloride, all at pH 7.0 ± 0.1. Chondroitinase activity eluted in the 0.25 M sodium chloride fraction.

This fraction was further purified by diluting the chondroitinase containing fraction

two-fold with 0.01 M sodium phosphate and applying the material onto a column containing cellufine sulfate (2.6 cm i.d. x 100 cm, Amicon) and eluting at a linear flow rate of 1.88 cm•min^{-1} with a linear gradient of sodium chloride, 0.0 to 0.4 M. Chondroitinase AC primarily eluted at 0.23 to 0.26 M sodium chloride while chondroitinase B eluted at 0.27 to 0.3 M sodium chloride.

Each fraction was diluted two-fold with 0.01 M sodium phosphate and applied to a hydroxylapatite column (2.6 cm i.d. x 30 cm). The bound proteins were eluted with a step gradient of 0.25 M sodium chloride followed by a linear gradient of 0.25 to 1.0 M sodium chloride all in 0.025 M sodium phosphate at pH 7.7 $\pm$ 0.1. Chondroitinase B elutes in the 0.25 M sodium chloride step while chondroitinase AC elutes at 0.85 to 0.95 M sodium chloride.

The chondroitinase B fraction was diluted two-fold in 0.01 M sodium phosphate and applied to a strong cation exchange column (CBX-S, J.T. Baker, 1.6 cm i.d. x 10 cm). The bound material was eluted at a flow rate of 1.0 cm•min^{-1} with a linear gradient from 0.125 to 0.325 M sodium chloride in 0.025 M sodium phosphate at pH 7.0 $\pm$ 0.1. Chondroitinase B eluted in a protein peak at 0.175 to 0.225 M sodium chloride and contained a minor contaminating protein of molecular weight 20,000 Daltons. This protein was removed by gel filtration chromatography by loading the chondroitinase B sample onto a Superdex™ 200 column (1.0 cm i.d. x 30 cm, Pharmacia) and eluting with 0.05 M sodium phosphate, pH 7.2 at a linear flow rate of 1.25 cm•min^{-1} and collecting the protein containing fractions.

The chondroitinase AC fraction collected from hydroxylapatite chromatography was diluted

three-fold in 0.01 M sodium phosphate and applied to a strong cation exchange column (CBX-S, J.T. Baker, 1.6 cm i.d. x 10 cm). The bound material was eluted at a flow rate of 1.0 cm•min^{-1} with a linear gradient from 0.125 to 0.325 M sodium chloride in 0.025 M sodium phosphate at pH 7.0 ± 0.1. Chondroitinase AC eluted in a single protein peak at 0.175 to 0.225 M sodium chloride. Purification results for the chondroitinase enzymes are shown in Table 1.

Table 1: Purification of chondroitinase enzymes from *Flavobacterium heparinum* fermentations

sample	activity (IU)	specific activity (IU/mg)	yield (%)
<u>fermentation:</u>			
chondroitinase AC	65,348	0.764	100
chondroitinase B	21,531	0.252	100
<u>osmolate:</u>			
chondroitinase AC	39,468	1.44	60
chondroitinase B	15,251	0.588	71
<u>cation exchange:</u>			
chondroitinase AC	27,935	9.58	43
chondroitinase B	13,801	4.731	64
<u>cellufine sulfate:</u>			
chondroitinase AC	18,160	22.6	28
chondroitinase B	6,274	21.2	29
<u>hydroxylapatite:</u>			
chondroitinase AC	14,494	146.8	22
chondroitinase B	3,960	65.62	18
<u>strong cation exchange:</u>			
chondroitinase AC	9,843	211.4	15
chondroitinase B	4,104	167.2	18
<u>gel filtration:</u>			
chondroitinase B	2,814	278.7	13

Chondroitinase activity was determined by a modification of the spectrophotometric assay described by Yang, et al., *J. Biol. Chem.*, 160(30):1849-1857, 1985. Chondroitinases degrade

their respective substrates by an eliminative reaction resulting in the formation of 4,5-unsaturated sulfated disaccharides which absorb ultraviolet light at 232 nm. Reaction buffers contained 50 mM Tris, pH 8.0 and 0.5 mg/ml substrate; dermatan sulfate for chondroitinase B activity, chondroitin sulfate A for chondroitinase AC activity. A continuous spectrophotometric assay is carried out by transferring a 10 to 50 μl sample to a quartz cuvette and adding the reaction buffer to make a final volume of one ml. The cuvette is placed in a Beckman DU 640 spectrophotometer, controlled to maintain a constant temperature of 30°C, and the increase in absorbance at 232 nm monitored for three to five minutes. Activities are calculated using the molar extinction coefficient for chondroitin sulfate, $5.1 \times 10^3\ M^{-1}$, and are expressed in international units, IU, where one IU is the amount of enzyme required to catalyze the formation of one μmole unsaturated product per minute.

<u>**Properties of Chondroitinase Enzymes**</u>

The purification method described herein is suitable for obtaining sufficient quantities of purified chondroitinase AC and chondroitinase B for characterization studies. The purified enzymes were analyzed by SDS-PAGE using the technique of Laemmli, *Nature*, 227:680-685, 1970, and the gels quantified with a scanning densitometer (Bio-Rad, Model GS-670). Chondroitinase AC was shown to have a molecular weight of 77,000 ± 5,000 Daltons and a purity of greater than 99% while chondroitinase B has a molecular weight of 55,000 ± 2,300 Daltons and a purity of greater than 99%.

Kinetic parameters of the 77,000 Dalton chondroitinase AC protein were measured using both chondroitin sulfate A and chondroitin sulfate C as

substrates. The K_m and K_{cat} values for chondroitinase A activity were 6 μM and 230 s^{-1}, respectively, while the K_m and K_{cat} values for chondroitinase C activity were 9.3 μM and 150 s^{-1}, respectively. Kinetic parameters of the 55,000 Dalton chondroitinase B protein were measured using dermatan sulfate as the substrate. The K_m and K_{cat} values for chondroitinase B activity were 7.4 μM and 192 s^{-1}, respectively.

Effect of Added Reagents

The V_{max} of the chondroitinase enzymes can be effected by trace amounts of certain elements. A base reaction buffer of 20 mM Tris buffer, pH 8.0 and 0.5 mg/ml substrate, either chondroitin sulfate A for chondroitinase AC or dermatan sulfate for chondroitinase B, was used to determine the effect of divalent metals and salts on the activity of the chondroitinase enzymes. The results are shown in Table 2.

Table 2: Effects of 0.1 mM of various reagents on the activity of chondroitinase enzymes.

reagent	chondroitinase AC relative activity(%)	chondroitinase B relative activity(%)
none	100	100
$MgCl_2$	91	91
$MnCl_2$	83	33
$CuSO_4$	92	91
$ZnCl_2$	26	45
$FeSO_4$	98	69
$HgCl_2$	55	40
$CoCl_2$	81	42
EDTA	97	1

Stabilization of Chondroitinases

The chondroitinase enzyme activity can be stabilized by addition of excipients or by lyophilization. Stabilizers include carbohydrates, amino acids, fatty acids, and surfactants and are known to those skilled in the art. Examples

include carbohydrate such as sucrose, lactose, mannitol, and dextran, proteins such as albumin and protamine, amino acids such as arginine, glycine, and threonine, surfactants such as Tween™ and Pluronic™, salts such as calcium chloride and sodium phosphate, and lipids such as fatty acids, phospholipids, and bile salts. The stabilizers are generally added to the protein in a ratio of 1:10 to 4:1, carbohydrate to protein, amino acids to protein, protein stabilizer to protein, and salts to protein; 1:1000 to 1:20, surfactant to protein; and 1:20 to 4:1, lipids to protein. Other stabilizers include high concentrations of ammonium sulfate, sodium acetate or sodium sulfate, based on comparative studies with heparinase activity. The stabilizing agents, preferably the ammonium sulfate or other similar salt, are added to the enzyme in a ratio of 0.1 to 4.0 mg ammonium sulfate/IU enzyme.

The use of stabilizers is demonstrated as follows. The purified chondroitinase enzymes were dialyzed into 10 mM sodium phosphate, pH 7.5, to a concentration of 2 IU/ml and supplemented with either 1 mg/ml bovine serum albumin, 1.5 M sodium acetate, 0.0025 M Tris or 0.15 M Tris, and an accelerated shelf life performed at 37°C. 2 IU of purified chondroitinase enzymes also were placed into various buffers, lyophilized and an accelerated shelf life performed at 37°C. The results are shown in Table 3.

Table 3: Stability of chondroitinase enzymes at 37°C.
7 day retention of activity (%)

additive	format	chondroitinase AC		chondroitinase B
0.15 M Tris	liquid	1	42	
0.0025 M Tris	liquid	22	44	
1 mg/ml BSA	liquid	1	26	
1.5 M NaOAc	liquid	64	72	
0.15 M Tris	lyophilized		26.7	43.7
PBS	lyophilized		8.7	15.9
8 mg/ml sucrose	lyophilized		88	93.16
2 mg/ml glycine	lyophilized		42.4	75.7

Cloning of Chondroitinase AC and Chondroitinase B

Amino Acid Analysis

The purified proteins were analyzed by the technique of Edman, *Ann. N. Y. Acad. Sci.* 88:602, 1950, to determine the N-terminal amino acid. However, the Edman chemistry was unable to liberate an amino acid, indicating that a post-translational modification had occurred at the N-terminal amino acid of both chondroitinase proteins. One nmol samples of chondroitinases AC and B were used for deblocking with pyroglutamate aminopeptidase. Control samples were produced by mock deblocking 1 nmol samples without adding the peptidase. All samples were placed in 10 mM ammonium carbonate buffer at pH 7.5 with 10 mM dithiothreitol. 1 mU peptidase was added to the samples and the reaction allowed to incubate at 37°C for 8 hours. An additional 0.5 mU peptidase was added and incubation continued for 16 h. The reaction mixture was exchanged into 35 % formic acid by diafiltration with 10,000 Dalton cut-off ultrafiltration membranes (Centricon, Amicon) and the sample dried under vacuum. Deblocked chondroitinase enzymes were then analyzed by Edman chemistry to determine the N-terminal sequence, using an Applied Biosystems 745A Protein Sequencer.

The N-terminal sequence of chondroitinase AC was QTGTAEL (Sequence ID No. 2, amino acids 24 to 30) and of chondroitinase B was VVASNEL (Sequence ID No. 4, amino acids 27 to 34).

The chondroitinase enzymes were subjected to enzymatic fragmentation using the arginine specific protease clostripain (EC 3.4.22.8, Sigma). Pre-activated clostripain was added to chondroitinase AC at a 1 to 2 % w/w ratio in 0.025 M sodium phosphate, 0.0002 M calcium acetate and 0.0025 M dithiothreitol at pH 7.5 ± 0.1 and incubated for 2 to 3 hours at 37°C. The reaction mixture was applied to a Vydac C_{18} reverse phase HPLC column (0.46 cm I.D. x 30 cm) and the peptide fragments eluted at a linear flow rate of 1 cm-min^{-1} with a linear gradient of 10 to 90 % acetonitrile in 1 % trifluoroacetic acid. Four of the peptide fragments obtained were subjected to amino acid sequence determination.

Clostripain was added to chondroitinase B at a 1 to 2 % w/w ratio in 0.025 M sodium phosphate, 0.0002 M calcium acetate and 0.0025 M dithiothreitol at pH 7.5 ± 0.1 and incubated for 2 to 3 hours at 37°C. The reaction mixture was applied to a Vydac™ C_{18} reverse phase HPLC column and the peptide fragments eluted at a linear flow rate of 6.0 cm°min^{-1} with a linear gradient of 10 to 90 % acetonitrile in 1 % trifluoroacetic acid. Three of the peptide fragments obtained were subjected to amino acid sequence determination.

Construction of *Flavobacterium heparinum* gene library

A *Flavobacterium heparinum* chromosomal DNA library was constructed in lambda phage DASHII. 0.4 µg of *F. heparinum* chromosomal DNA was partially digested with restriction enzyme, *Sau*3A, to produce a majority of fragments around 20 kb in size, as described in Maniatis, *et al.*, *Molecular Cloning, A*

laboratory Manual, 1982. This DNA was phenol/chloroform extracted, ethanol precipitated, ligated with DASHII arms and packaged with packaging extracts from a Lambda DASHII™/*Bam*HI Cloning Kit (Stratagene, La Jolla, CA). The library was titered at approximately 10^{-5} pfu/ml after packaging, was amplified to 10^{-8} pfu/ml by the plate lysis method, and stored at -70°C as described by Silhavy *et al.* in *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory, 1972.

The *F. heparinum* chromosomal library was titered to about 300 pfu/plate, overlaid on a lawn of *E. coli*, and allowed to transfect the cells overnight at 37°C, forming plaques. The phage plaques were transferred to nitrocellulose paper, and the phage DNA bound to the filters, as described in Maniatis, *et al.*, *ibid*.

Nucleic acid sequence encoding Chondroitinase AC

Degenerate primers were designed from peptides AC-1, AC-3 and AC-4 (Sequence ID No. 2, amino acids 395 to 413; 603 to 617; 514 to 536; and 280 to 288, respectively). Amplification of the primers was carried out in a 0.1 ml reaction buffer containing 50 mM KCl, 10 mM Tris/HCl pH 9, 0.1% Triton X-100, 2.5 mM $MgCl_2$, plus the four dNTPs at 200 μM, 2.5 units Taq Polymerase (Bio/Can, Mississauga, Ont.), 0.1 mM of each primer and 10 ng of *F. heparinum* genomic DNA. The amplified primers were linearized with *Sal*I, *Not*I, and *Xba*I in individual restriction digests, and combined, after purification, for use as template DNA. The samples were placed in an automated heating block, (DNA Thermocycler™, Barnstead/Thermolyne, Dubuque, IA) programmed for cycles with temperatures of denaturation at 94°C for 1 min., annealing at 50°C for 2 min., and extension at 72°C for 2 min., with 35 repetitions of this sequence. The combination of

synthetic oligonucleotide primers: 5'-TCNGGRAARTARTANCCDATNGCRTCRTG-3' (Sequence ID No. 5), corresponding to peptide AC-3; and 5'-TAYATGGAYTTYAAYGTNGARGG-3' (Sequence ID No. 6), corresponding to peptide AC-4; yielded a PCR product of approximately 750 bp in size. Attempts to clone this fragment into vectors, pTZ/PC or into pCRII (TA cloning kit, Invitrogen, San Diego, Ca.) in *E. coli* strain, FTB1, were unsuccessful.

E. coli FTB1 was constructed as follows: the F' episome from *E. coli* XL-1 Blue, (Stratagene, La Jolla CA) carrying the *lac* I^q repressor gene was moved, as described by Miller, *Experiments in Molecular Genetics*, Cold Spring Harbor, 1972, into *E. coli* TB1 described by Baker *et al.*, *Proc. Natl. Acad. Sci.* 81:6779-6783, 1984. The FTB1 background permits a more stringent repression of transcription from plasmids carrying promoters with a *lac* operator such as the *lac* and tac promoters.

To facilitate cloning of these PCR products, a restriction site was incorporated at the 5' ends of the primers. The PCR products were analyzed for the absence of restriction sites which are found in the multiple cloning site of pBluescript (Stratagene, La Jolla, CA) to determine which restriction site should be added to the primers. This ensured that the PCR products would not be cut into multiple fragments when treated with the restriction enzyme used to form overhangs on the ends of the DNA fragments. *Bam*HI met this criteria for all three PCR fragments. New primers were synthesized with *Bam*HI sites at their 5' ends, which were otherwise identical to those described above, and used to produce a 764 bp PCR product, Figure 1. This DNA fragment was digested with *Bam*HI, isolated on an agarose gel, as described by Maniatis *et al.*, *ibid*, and purified using the

Geneclean™ kit (Bio/Can, Mississauga, Ont.) pBluescript was digested with *Bam*HI, the 5′ ends dephosphorylated by alkaline phosphatase treatment as described by Maniatis et al., *ibid*, and purified from an agarose gel using the Geneclean™ kit. The treated PCR fragment and pBluescript plasmid DNA were ligated, transformed into FTB1, and plated onto LB agar plates containing ampicillin at 0.2 mg/ml. Plasmids from colonies grown on these plates were isolated by colony cracking as described in Maniatis *et al.*, *ibid*. All enzymes were supplied by New England Biolabs (Mississauga, Ont.). Plasmids were isolated using the RPM™ kit (Bio/Can, Mississuaga, Ont.). Sequence analysis of the cloned PCR fragment correlated with reverse transcribed peptide sequences from chondroitinase AC peptides, indicating that the PCR fragment encodes the chondroitinase AC gene. DNA sequencing was performed by the dideoxy-chain termination method of Sanger *et al.*, *Proc. Natl. Acad. Sci.*, 74:5463-5467, 1978. Sequencing reactions were carried out with the Sequenase™ Kit (U.S. Biochemical Corp., Cleveland, Ohio) and S-dATP (Amersham Canada Ltd., Oakville, Ontario, Canada), as specified by the supplier.

The 764 bp PCR fragment, contained in plasmid pA2C1BS-11 represents approximately 36% of the coding region for the Chondroitinase AC gene. This entire 764 bp fragment was sequenced and was found to contain a continuous open reading frame which encoded peptides AC-3, AC-4 and AC-1 (Sequence ID No. 2, amino acids 395-413; 603-617; 514-536; 280-288, respectively).

The 764 bp PCR fragment was used to probe the genomic *F. heparinase* lambda library. First, pA2C1BS-11 was isolated via the boiling method, as described in Maniatis *et al.*, *ibid*. The plasmid

was digested with *Bam*HI, separated from the vector, purified as described above and labeled with a Nick Translation™ kit (Boehringer Mannheim, Montreal, Canada) using radiolabelled ^{32}P α-dATP. *E. coli* P2392 (Stratagene, La Jolla, CA) was used as the lawn for plating the lambda library. Approximately 6000 plaques were screened by plaque hybridization using BA85 nitrocellulose membranes (Scheicher & Schuell, Keene, NH) as described by Maniatis *et al.*, *ibid.* Plaque hybridization was carried out, at 65°C for 16 hours in a Tek Star™ hybridization oven (Bio/CAN Scientific, Mississauga, Ontario). Subsequent washes were performed at 65°C, twice for 15 min. in 2X SSC, once in 2X SSC/0.1% SDS for 30 min. and once in 0.5X SSC/0.1% SDS for 15 min. More than 100 positive plaques were identified and isolated, some of which were clusters of plaques. These were rescreened by spotting the lambda clone onto a lawn of P2392 host cells and reprobing via plaque hybridization. Six plaques were positive upon rescreening, and their DNA was isolated, as described by Maniatis, *et al.*, *ibid*, and digested with restriction enzymes corresponding to the sites on the ends of lambda DASH II arms. This DNA was used in Southern hybridization analysis (Southern, *J. Mol. Biol.* 98:503-517, 1975) by blotting onto Hybond™ N nylon membrane (Amersham, Oakville, Canada) using hybridization and wash conditions, described above for plaque hybridization. One clone contained a 4.5 kb Sal I fragment and another contained a 6 kb *Bam*HI fragment, both of which hybridized with the probe. These were cloned into corresponding sites of pBluescript.

Because the molecular weight of chondroitinase AC is approximately 75 kD, the size of the corresponding gene would be approximately 2.05 kb. Both the 4.5 kb *Sal*I and the 6 kb *Bam*HI

chromosomal DNA fragments could include the entire chondroitinase AC gene. To increase the probability of analyzing a DNA fragment which encodes the entire gene, the 6 kb *Bam*HI fragment was chosen for sequence analysis. The pBluescript plasmid containing this *Bam*HI fragment (called p64BS2-7, Figure 1) was isolated using the Qiagene kit (Bio/Can, Miss, Ont). A method of DNA sequencing, the walking primer strategy (Voss *et al. Meth. Molec. Cell. Biol. 3*:153-155 (1992)), was employed using synthetic primers (Eppendorf, model ECOSYN™ D300,Madison, WI) and an A.L.F. DNA sequencer (Pharmacia LKB, Mtl, Qc). Fluorescenated Universal and Reverse primers provided in the Pharmacia AutoRead kit were also used. Fluorescently labeled dNTPs were incorporated into sequencing reactions with the Pharmacia AutoRead Fluorescent labelling kit (Pharmacia LKB, Mtl, QC). Areas of secondary structure were resolved by one of two methods. First, fluorescenated primers which hybridized close to, and 5′ to, the region of secondary structure were synthesized. Using these primers, the Pharmacia AutoCycle™ kit (Pharmacia LKB, Mtl, Qc), and a automated heating block (DNA Thermocycler™, Barnstead/Thermolyne, Dubuque, Iowa), programmed for step cycles of 95°C for 36 sec, 50°C for 36 sec and 72°C for 84 sec, repeated 25 times, sequencing of secondary structure regions was accomplished. Any ambiguous areas still not resolved by the first method were sequenced by the method of Sanger *et al., Proc. Natl. Acad. Sci. 74*: 5463-5467 (1978), using ^{35}S a-dATP, and a USB Sequenase™ kit (LaJolla, Ca.) in which dGTP was replaced by dITP.

Analysis of the DNA sequence indicated that there was a single, continuous open reading frame of 2100 bp containing codons for 700 amino acid

residues. All four clostropain-derived peptides were encoded by this gene. Searching for a possible signal peptide sequence using Geneworks™ (Intelligenetics, Mountain View, Ca.), suggested that there are two possible sites for the processing of the protein into a mature form: Q-23 (glutamine) and A-28 (alanine). N-terminal amino acid sequencing of deblocked, processed Chondroitinase AC indicated that the mature protein begins with Q-23 and contains 678 amino acids with a calculated molecular weight of 77,169 Daltons.

Expression of Chondroitinase AC in *E. coli*

Construction of an expression vector for chondroitinase AC is shown in Figure 2. The vector pGB is an *E. coli* expression vector which contains an unique *BamHI* site, whereby expression of a DNA fragment inserted into this site is driven by a double tac promoter. The vector also includes a kanamycin resistance gene and the *lac* I^q gene to allow induction of transcription with IPTG. PCR was used to generate a mature chondroitinase AC gene.

An oligonucleotide, 5'-GCGGATCCATGCAGCAGACCGGTACTGCAGAA-3', (Sequence ID No. 7) was designed to insert an ATG-start site immediately preceding the codon for the first amino acid (Q-23) of mature chondroitinase AC, while an oligonucleotide 5'-CGCGGATCCCCTAGATTACTACCATCAAAA-3' (Sequence ID No. 8) was designed to hybridize downstream of the TAG-stop codon. Both oligonucleotides also contain a *Bam*HI site. Plasmid p64BS2-7 was used as the template in a PCR reaction with an annealing temperature of 45°C. A specific fragment of the expected size of 2034 bp was obtained. This fragment was isolated and inserted into a *Bam*HI site of the expression vector pGB.

The construct was transformed into *E. coli* strain, F-TB1, and the transformed bacteria was grown at 37°C in LB medium containing 75 μg/ml kanamycin to an OD_{600} of 0.5, at which point the tac promoter from pGB was induced by the addition of 1 mM IPTG. Cultures were grown an additional 2 to 5.5 hours at either 23°C, 30°C or 37°C. The cells were cooled on ice, concentrated by centrifugation and resuspended in cold PBS at 1/10th the original culture volume. Cells were lysed by sonication and cell debris removed by centrifugation at 10,000 x g, 5 minutes. The pellet and supernatant fractions were analyzed separately for chondroitin sulfate A or C degrading (chondroitinase AC) activity. Chondroitin sulfate A degrading activities of 1.24 x 10^{-2}, 2.88 x 10^{-2}, and 4.25 x 10^{-2} IU/ml/OD and chondroitin sulfate C degrading activities of 1.57 x 10^{-2}, 2.24 x 10^{-2}, and 6.02 x 10^{-2} IU/ml/OD were observed from cultures grown at 23, 30 and 37°C, respectively. The activities using chondroitin sulfate A as the substrate are approximately twice that of those using chondroitin sulfate C as the substrate. This ratio is also observed when measuring the activity of the wild type chondroitinase AC using both these substrates.

E. coli F-TB1(pGB-ChAC) was grown in a 3.5 L Braun Biostat E computer controlled fermenter in M9 medium to a dry cell weight concentration of 35 g/L. Glucose and ammonia were added as needed to maintain growth and pH at 7.0. Chondroitinase A activity accumulated to 103.44 IU/ml while chondroitinase C activity accumulated to 28.26 IU/ml.

Nucleic Acid encoding Chondroitinase B

Partial-guessmer PCR primers were designed using the amino acid sequences of the clostripain-generated peptides from the chondroitinase B

protein and the codons commonly found in *Flavobacterium* genes, Table 4. Three peptides were generated, designated CHB-1 (Sequence ID No. 4, amino acids 373 to 384), CHB-2 (Sequence ID No. 4, amino acids 41 to 50), and CHB-3 (Sequence ID No. 4, amino acids 130 to 146).

Table 4: Codon usage table for *Flavobacterium* and *Escherichia coli*.

amino acid	codon(s)	consensus codon *E. coli*	consensus codon *Flavobacterium*
A	GCT, GCC, GCG, GCA	GCT	GCC
C	TGT,TGC	EITHER	EITHER
D	GAT, GAC	EITHER	EITHER
E	GAG, GAA	GAA	GAA
F	TTC, TTT	EITHER	TTT
G	GGC, GGA, GGG, GGT	GGC or GGT	GGC
H	CAC, CAT	CAT	CAT
I	ATC, ATA, ATT	ATA	ATC
K	AAA, AAG	AAA	AAA
L	CTT, CTA, CTG, TTG, TTA, CTC	CTG	CTG
M	ATG	ATG	ATG
N	AAC, AAT	AAC	AAT
P	CCC, CCT, CCA, CCG	CCG	CCG
Q	CAG, CAA	CAG	CAG
R	CGT, AGA, CGC, CGA, AGG, CGG	CGT	CGC
S	TCA, TCC, TCG, TCT, AGC, AGT	TCT	ND
T	ACG, ACC, ACT, ACA	ACC or ACT	ACC or ACA
V	GTC, GTA, GTT, GTG	GTT	ND
W	TGG	TGG	TGG
Y	TAC, TAT	EITHER	TAT

5'-CGG GAT CCC ARA TYG CCG AYG GNA CNT ATA AAG A-3' (Sequence ID No. 9) was derived from the CHB-2 peptide (Sequence ID No. 4, amino acids 41 to 50) and 5'-CGG GAT CCG GCN SKA TTG CGT TCR TCA AA-3' (Sequence ID No. 10) was derived from peptide CHB-3, Sequence ID No. 4, amino acids 130 to 146. A *Bam*HI site was present on the 5' end of each primer to increase the efficiency of cloning of the PCR product. Using linear *F. heparinum* chromosomal DNA, described above, as a template, a single 300 bp DNA fragment was amplified. Conditions for the amplification were as follows:

denaturation at 94°C for 40 sec, annealing at 45 or 50°C for 1 min. and extension at 72°C for 2 min. This cycle was repeated 35 times.

As shown in Figure 3, the PCR fragment was purified on an agarose gel, digested with *Bam*HI and ligated into *Bam*HI digested, dephosphorylated pBluescript. The ligation mixture was used to transform *E. coli* FTB1. Of the 50 resulting transformants, one yielded a 300 bp fragment when cut with *Bam*HI. The insert in this plasmid, pCHB300, was subjected to DNA sequence analysis, performed as described above, which revealed that the insert contained DNA sequences outside of the primer regions which encoded amino acid sequence matching that determined for two chondroitinase B peptides. This insert was used to screen the lambda library of *F. heparinum* chromosomal DNA, which was constructed as described above.

The lambda library was plated with a density of 200 plaques per dish. Plate lifts of 20 dishes were made. For production of the probe, 500 ng of pCHB300 was submitted to 30 cycles of PCR amplification; denaturation at 93°C, annealing at 55°C and extension at 72°C, each for 1 min., using the primers described above. The resulting PCR fragment was purified on agarose gels and labelled with dATPa^{32}P, using the Random Primer labelling kit (Boehringer Mannheim, Laval, Canada). Thirty-one potential lambda clones were found which hybridized with this probe, after the lifts were subjected to washing one time, in 2X SSC at 58°C. Rescreening of these plaques gave a positive signal for 17 of the plaques after washing at 58°C, 2X for 15 min. in 2X SSC, 1X for 30 min. in 2X SSC/0.1% SDS and 1X for 20 min. in 0.5X SSC/0.1% SDS. Two of 8 clones analyzed further showed a 5.0 kb *Hin*dIII fragment hybridizing with the probe and comigrating with a

*Hin*dIII fragment from *F. heparinum* chromosomal DNA which also hybridized with the 300 bp probe. The 5.0 kb fragment was gel purified from both lambda clones, ligated into the *Hin*dIII site of pBluescript and transformed into FTB1.

44 colonies were picked and rubbed on the side of a 0.5 ml PCR tube containing 20 μl of the same PCR mixture as above. PCR was performed at: denaturation at 93°C, for 30 sec., annealing at 58°C, for 30 sec. and extension at 72°C, for 1 min, for 35 cycles. Upon analysis, 6 transformants showed amplification of the 300 bp band. DNA from these colonies were isolated and digested by *Hin*dIII revealing the presence of a 5.0 kb fragment. 5 out of the 6 clones hybridized with the 300 bp fragment, confirming results of the PCR amplification experiment. One of these clones, pCHB78, was selected and used as a template for DNA sequencing.

Using a walking primer strategy, sequencing reactions were carried out as described above for the A.L.F. DNA sequencer. Sequence analysis revealed a single 1.52 kb open reading frame coding for 506 amino acid residues. The preprotein was found to have a signal peptide of 25 amino acids. The mature chondroitinase B enzyme contains 481 amino acids with a calculated molecular weight of 53,563 daltons.

Expression of Chondroitinase B in *E. coli*

Construction of an expression vector for chondroitinase B is shown in Figure 4. Primers were designed to amplify the coding region of the chondroitinase B gene in an analogous manner to that described above with reference to expression of the chondroitinase AC gene. One oligonucleotide used for amplification of the chondroitinase B coding sequence (5'-

CGCGGATCCATGCAGGTGTTGCTCAAATGAAACT-3') (Sequence ID No. 11), contained a *Bam*HI restriction site at its 5' end and an ATG codon that was to be inserted before the first amino acid of the mature protein. The second oligonucleotide (5'-CGGAATCAATTCACCGGG-AT-3') (Sequence ID No. 12) was designed with a *Xmn*I restriction site and a termination codon to be inserted at the end of the coding sequence of the gene. Using 100 ng of pCHB78 as template, with an annealing temperature of 52°C, the 1.5 kb fragment was amplified, gel purified, restriction digested and inserted into pGB previously cut with *Bam*HI and *Xmn*I. This resulted in the definitive pGB-CHB construct used to express the protein.

This construct was transformed in *E. coli* strain DH5α, expressed as described for the chondroitinase AC enzyme. After growing cells until an O.D. 600 = 0.5, 1 mM IPTG was added to the cultures to induce the tandem tac promoters and cells were transferred to either 23°C, 30°C or 37°C for additional growth for 5, 3 and 2 hours, respectively. After sonication, supernatant fractions were assayed for activity on dermatan sulfate. Growth of cells at 23°C gave the best results with a degrading activity of 0.57 IU/ml/OD while growth of cells at 30°C and 37°C gave degrading activities of 0.14 and 0.01 IU/ml/OD respectively.

The present invention describes a methodology for obtaining highly purified chondroitin degrading enzymes derived from the natural organism *Flavobacterium heparinum*, and the genes encoding these enzymes. Derivatives of the genes can be prepared by making conservative substitutions, additions and deletions thereof, which do not substantially impact on the resulting enzymatic activity, or by using degenerative forms

of the genes. As used herein, conservative substitutions involve substitutions of codons which encode the same amino acids and substitutions of amino acids for amino acids having similar structure or chemical characteristics, which are well known to those skilled in the art, for example, groups of structurally similar amino acids include (I,L,V); (F,Y); (K,R); (Q,N); (D,E); AND (G,A).

Variations of these methods will be obvious to those skilled in the art from the foregoing detailed description of the invention. Such modifications are intended to come within the scope of the appended claims.

SEQUENCE LISTING

```
(1) GENERAL INFORMATION:
     (i) APPLICANT: IBEX TECHNOLOGIES R AND D, INC.
    (ii) TITLE OF INVENTION: CHONDROITIN LYASE ENZYMES
   (iii) NUMBER OF SEQUENCES: 19
    (iv) CORRESPONDENCE ADDRESS:
          (A) ADDRESSEE: Patrea L. Pabst
          (B) STREET: 2800 One Atlantic Center, 1201 West Peachtree Street
          (C) CITY: Atlanta
          (D) STATE: Georgia
          (E) COUNTRY: USA
          (F) ZIP: 30309-3450
     (v) COMPUTER READABLE FORM:
          (A) MEDIUM TYPE: Floppy disk
          (B) COMPUTER: IBM PC compatible
          (C) OPERATING SYSTEM: PC-DOS/MS-DOS
          (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
  (viii) ATTORNEY/AGENT INFORMATION:
          (A) NAME: Pabst, Patrea L.
          (B) REGISTRATION NUMBER: 31,284
          (C) REFERENCE/DOCKET NUMBER: IT103
    (ix) TELECOMMUNICATION INFORMATION:
          (A) TELEPHONE: (404) 873-8794
          (B) TELEFAX: (404) 873-8795
(2) INFORMATION FOR SEQ ID NO:1:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 2103 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: DNA (genomic)
   (iii) HYPOTHETICAL: NO
    (iv) ANTI-SENSE: NO
    (ix) FEATURE:
          (A) NAME/KEY: misc_feature
          (B) LOCATION: 1..2103
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(D) OTHER INFORMATION: /note= "Nucleic acid sequence encoding chondroitinase AC from Flavobacterium heparinum."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AAGCCTTTGC GCAATATGGA TAAGGTGGCG GAAAAGAACC TGAATACGCT GCAGCCTGAC    180
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CACCTGCTAC AATTGGAAAC TATTATACAG GCTTATATTG AAAAAGATAG TCACTATTAT    300
GGCGACGATA AAGTGTTTGA CCAGATTTCC AAAGCTTTTA AGTATTGGTA TGACAGCGAC    360
CCGAAAAGCC GCAACTGGTG GCACAATGAA ATTGCCACTC CGCAGGCCCT TGGTGAAATG    420
CTGATCCTGA TGCGTTACGG TAAAAAGCCG CTTGATGAAG CATTGGTGCA TAAATTGACC    480
GAAAGAATGA AGCGGGGCGA ACCGGAGAAG AAAACGGGGG CCAACAAAAC AGATATCGCC    540
CTGCATTACT TTTATCGTGC TTTGTTAACG TCTGATGAGG CTTTGCTTTC CTTCGCCGTA    600
AAAGAATTGT TTTATCCCGT ACAGTTTGTA CACTATGAGG AAGGCCTGCA ATACGATTAT    660
TCCTACCTGC AGCACGGTCC GCAATTACAG ATATCGAGCT ACGGTGCCGT ATTTATTACC    720
GGGGTACTGA AACTTGCCAA TTACGTTAGG GATACCCCTT ATGCTTTAAG TACCGAGAAA    780
CTGGCTATAT TTTCAAAGTA TTACCGCGAC AGTTATCTGA AAGCTATCCG TGGAAGTTAT    840
ATGGATTTTA ACGTAGAAGG CCGCGGAGTA AGCCGGCCAG ACATTCTAAA TAAAAAGGCA    900
GAAAAAAAGA GGTTGCTGGT GGCGAAGATG ATCGATCTTA AGCATACTGA AGAATGGGCT    960
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GATGCGATAG CCAGGACAGA TAGCACAGTT GCGGCCGGCT ATAAGATTGA GCCCTATCAC   1020
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CGTATGGTGA GTAAGCGGAC CCGACGCAGT GAATCCGGCA ATAAAGAAAA CCTGCTGGGC   1140
AGGTATTTAT CTGATGGGGC TACTAACATA CAATTGCGCG GACCAGAATA CTATAACATT   1200
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GCCTGGTTCT TTTTTGACAA AGAGATTGTA TGTCTTGGTG CCGGTATCAA CAGCAATGCC   1440
CCTGAAAACA TTACCACTAC CCTTAACCAG AGCTGGTTAA ATGGCCCGGT TATAAGTACT   1500
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TCGCAAAAAG GCAATTGGTT CCACATCAAC AATTCACATT CAAAAGATGA AGTTTCTGGT   1680
GATGTATTTA AGCTTTGGAT CAACCATGGT GCCAGGCCAG AAAATGCGCA GTATGCTTAT   1740
ATCGTTTTGC CGGGAATAAA CAAGCCGGAA GAAATTAAAA AATATAATGG AACGGCACCG   1800
AAAGTCCTTG CCAATACCAA CCAGCTGCAG GCAGTTTATC ATCAGCAGTT AGATATGGTA   1860
CAGGCTATCT TCTATACAGC TGGAAAATTA AGCGTAGCGG GCATAGAAAT TGAAACAGAT   1920
AAGCCATGTG CAGTGCTGAT CAAGCACATC AATGGCAAGC AGGTAATTTG GGCTGCCGAT   1980
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AATCGGGTAA AAATTGATTT TCCGCAACAG GAATTTGCAG GTGCAACGGT TGAACTGAAA    2100

TAG                                                                  2103

(2) INFORMATION FOR SEQ ID NO:2:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 700 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: protein
    (ix) FEATURE:
          (A) NAME/KEY: Peptide
          (B) LOCATION: 1..23
          (D) OTHER INFORMATION: /note= "Amino acids 1 through 23 are a leader
peptide."
    (ix) FEATURE:
          (A) NAME/KEY: misc_feature
          (B) LOCATION: 1..700
          (D) OTHER INFORMATION: /note= "Amino acid sequence
                of chondroitinase AC from Flavobacterium
                heparinum."

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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                 20                  25                  30
     Lys Arg Val Met Leu Asp Leu Lys Lys Pro Leu Arg Asn Met Asp Lys
             35                  40                  45
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Val Ala Glu Lys Asn Leu Asn Thr Leu Gln Pro Asp Gly Ser Trp Lys
    50                  55                  60
Asp Val Pro Tyr Lys Asp Asp Ala Met Thr Asn Trp Leu Pro Asn Asn
65                  70                  75                  80
His Leu Leu Gln Leu Glu Thr Ile Ile Gln Ala Tyr Ile Glu Lys Asp
                85                  90                  95
Ser His Tyr Tyr Gly Asp Asp Lys Val Phe Asp Gln Ile Ser Lys Ala
            100                 105                 110
Phe Lys Tyr Trp Tyr Asp Ser Asp Pro Lys Ser Arg Asn Trp Trp His
        115                 120                 125
Asn Glu Ile Ala Thr Pro Gln Ala Leu Gly Glu Met Leu Ile Leu Met
    130                 135                 140
Arg Tyr Gly Lys Lys Pro Leu Asp Glu Ala Leu Val His Lys Leu Thr
145                 150                 155                 160
Glu Arg Met Lys Arg Gly Glu Pro Glu Lys Lys Thr Gly Ala Asn Lys
                165                 170                 175
Thr Asp Ile Ala Leu His Tyr Phe Tyr Arg Ala Leu Leu Thr Ser Asp
            180                 185                 190
Glu Ala Leu Leu Ser Phe Ala Val Lys Glu Leu Phe Tyr Pro Val Gln
        195                 200                 205
Phe Val His Tyr Glu Glu Gly Leu Gln Tyr Asp Tyr Ser Tyr Leu Gln
    210                 215                 220
His Gly Pro Gln Leu Gln Ile Ser Ser Tyr Gly Ala Val Phe Ile Thr
225                 230                 235                 240
Gly Val Leu Lys Leu Ala Asn Tyr Val Arg Asp Thr Pro Tyr Ala Leu
                245                 250                 255
Ser Thr Glu Lys Leu Ala Ile Phe Ser Lys Tyr Tyr Arg Asp Ser Tyr
            260                 265                 270
Leu Lys Ala Ile Arg Gly Ser Tyr Met Asp Phe Asn Val Glu Gly Arg
        275                 280                 285
Gly Val Ser Arg Pro Asp Ile Leu Asn Lys Lys Ala Glu Lys Lys Arg
    290                 295                 300
Leu Leu Val Ala Lys Met Ile Asp Leu Lys His Thr Glu Glu Trp Ala
305                 310                 315                 320
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Asp Ala Ile Ala Arg Thr Asp Ser Thr Val Ala Ala Gly Tyr Lys Ile
                325                 330                 335
Glu Pro Tyr His His Gln Phe Trp Asn Gly Asp Tyr Val Gln His Leu
            340                 345                 350
Arg Pro Ala Tyr Ser Phe Asn Val Arg Met Val Ser Lys Arg Thr Arg
        355                 360                 365
Arg Ser Glu Ser Gly Asn Lys Glu Asn Leu Leu Gly Arg Tyr Leu Ser
    370                 375                 380
Asp Gly Ala Thr Asn Ile Gln Leu Arg Gly Pro Glu Tyr Tyr Asn Ile
385                 390                 395                 400
Met Pro Val Trp Glu Trp Asp Lys Ile Pro Gly Ile Thr Ser Arg Asp
                405                 410                 415
Tyr Leu Thr Asp Arg Pro Leu Thr Lys Leu Trp Gly Glu Gln Gly Ser
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Asn Asp Phe Ala Gly Gly Val Ser Asp Gly Val Tyr Gly Ala Ser Ala
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Tyr Ala Leu Asp Tyr Asp Ser Leu Gln Ala Lys Lys Ala Trp Phe Phe
    450                 455                 460
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465                 470                 475                 480
Pro Glu Asn Ile Thr Thr Thr Leu Asn Gln Ser Trp Leu Asn Gly Pro
                485                 490                 495
Val Ile Ser Thr Ala Gly Lys Thr Gly Arg Gly Lys Ile Thr Thr Phe
            500                 505                 510
Lys Ala Gln Gly Gln Phe Trp Leu Leu His Asp Ala Ile Gly Tyr Tyr
        515                 520                 525
Phe Pro Glu Gly Ala Asn Leu Ser Leu Ser Thr Gln Ser Gln Lys Gly
    530                 535                 540
Asn Trp Phe His Ile Asn Asn Ser His Ser Lys Asp Glu Val Ser Gly
545                 550                 555                 560
Asp Val Phe Lys Leu Trp Ile Asn His Gly Ala Arg Pro Glu Asn Ala
                565                 570                 575
Gln Tyr Ala Tyr Ile Val Leu Pro Gly Ile Asn Lys Pro Glu Glu Ile
            580                 585                 590
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Lys Lys Tyr Asn Gly Thr Ala Pro Lys Val Leu Ala Asn Thr Asn Gln
        595                 600                 605
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    610                 615                 620
Tyr Thr Ala Gly Lys Leu Ser Val Ala Gly Ile Glu Ile Glu Thr Asp
625                 630                 635                 640
Lys Pro Cys Ala Val Leu Ile Lys His Ile Asn Gly Lys Gln Val Ile
                645                 650                 655
Trp Ala Ala Asp Pro Leu Gln Lys Glu Lys Thr Ala Val Leu Ser Ile
            660                 665                 670
Arg Asp Leu Lys Thr Gly Lys Thr Asn Arg Val Lys Ile Asp Phe Pro
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Gln Gln Glu Phe Ala Gly Ala Thr Val Glu Leu Lys
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 1521 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
   (A) NAME/KEY: misc_feature
   (B) LOCATION: 1..1521
   (D) OTHER INFORMATION: /note= "Nucleotide sequence encoding chondroitinase B from flavobacterium heparinum. "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATGAAGATGC TGAATAAACT AGCCGGATAC TTATTGCCGA TCATGGTGCT GCTGAATGTG      60

GCACCATGCT TAGGTCAGGT TGTTGCTTCA AATGAAACTT TATACCAGGT TGTAAAGGAG     120

GTAAAACCCG GTGGTCTGGT ACAGATTGCC GATGGGACTT ATAAAGATGT TCAGCTGATT     180
```

```
GTCAGCAATT CAGGAAAATC TGGTTTGCCC ATCACTATTA AAGCCCTGAA CCCGGGTAAG    240
GTTTTTTTTA CCGGAGATGC TAAAGTAGAG CTGAGGGGCG AGCACCTGAT ACTGGAAGGC    300
ATCTGGTTTA AAGACGGGAA CAGAGCTATT CAGGCATGGA AATCACATGG ACCCGGATTG    360
GTGGCTATAT ATGGTAGCTA TAACCGCATT ACCGCATGTG TATTTGATTG TTTTGATGAA    420
GCCAATTCTG CTTACATTAC TACTTCGCTT ACCGAAGACG GAAAGGTACC TCAACATTGC    480
CGCATAGACC ATTGCAGTTT TACCGATAAG ATCACTTTTG ACCAGGTAAT TAACCTGAAC    540
AATACAGCCA GAGCTATTAA AGACGGTTCG GTGGGAGGAC CGGGGATGTA CCATCGTGTT    600
GATCACTGTT TTTTTTCCAA TCCGCAAAAA CCGGGTAATG CCGGAGGGGG AATCAGGATT    660
GGCTATTACC GTAATGATAT AGGCCGTTGT CTGGTAGACT CTAACCTGTT TATGCGTCAG    720
GATTCGGAAG CAGAGATCAT CACCAGCAAA TCGCAGGAAA ATGTTTATTA TGGTAATACT    780
TACCTGAATT GCCAGGGCAC CATGAACTTT CGTCACGGTG ATCATCAGGT GGCCATTAAC    840
AATTTTTATA TAGGCAATGA CCAGCGATTT GGATACGGGG GAATGTTTGT TTGGGGAAGC    900
AGGCATGTCA TAGCCTGTAA TTATTTTGAG CTGTCCGAAA CCATAAAGTC GAGGGGGAAC    960
GCCGCATTGT ATTTAAACCC CGGTGCTATG GCTTCGGAGC ATGCTCTTGC TTTCGATATG   1020
TTGATAGCCA ACAACGCTTT CATCAATGTA AATGGGTATG CCATCCATTT TAATCCATTG   1080
GATGAGCGCA GAAAAGAATA TTGTGCAGCC AATAGGCTTA AGTTCGAAAC CCCGCACCAG   1140
CTAATGTTAA AAGGCAATCT TTTCTTTAAG GATAAACCTT ATGTTTACCC ATTTTTTAAA   1200
GATGATTATT TTATAGCAGG GAAAAATAGC TGGACTGGTA ATGTAGCCTT AGGTGTGGAA   1260
AAGGGAATCC CTGTTAACAT TTCGGCCAAT AGGTCTGCCT ATAAGCCGGT AAAAATTAAA   1320
```

```
GATATCCAGC CCATAGAAGG AATCGCTCTT GATCTCAATG CGCTGATCAG CAAAGGCATT    1380

ACAGGAAAGC CCCTTAGCTG GGATGAAGTA AGGCCCTACT GGTTAAAAGA AATGCCCGGG    1440

ACGTATGCTT TAACGGCCAG GCTTTCTGCA GATAGGGCTG CAAAGTTTAA AGCCGTAATT    1500

AAAAGAAATA AAGAGCACTG A                                              1521
```

```
(2) INFORMATION FOR SEQ ID NO:4:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 511 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: protein
    (ix) FEATURE:
          (A) NAME/KEY: Peptide
          (B) LOCATION: 1..25
          (D) OTHER INFORMATION: /note= "Amino acids 1 through 25
                are a signal peptide."
    (ix) FEATURE:
          (A) NAME/KEY: misc_feature
          (B) LOCATION: 1..700
          (D) OTHER INFORMATION: /note= "Amino acid sequence of chondroitinase B from
              Flavobacterium heparinum."
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

     Met Lys Met Leu Asn Lys Leu Ala Gly Tyr Leu Leu Pro Ile Met Val
     1               5                   10                  15
     Leu Leu Asn Val Ala Pro Cys Leu Gly Gln Val Val Ala Ser Asn Glu
                 20                  25                  30
```

```
Thr Leu Tyr Gln Val Val Lys Glu Val Lys Pro Gly Gly Leu Val Gln
        35                  40                  45
Ile Ala Asp Gly Thr Tyr Lys Asp Val Gln Leu Ile Val Ser Asn Ser
    50                  55                  60
Gly Lys Ser Gly Leu Pro Ile Thr Ile Lys Ala Leu Asn Pro Gly Lys
65                  70                  75                  80
Val Phe Phe Thr Gly Asp Ala Lys Val Glu Leu Arg Gly Glu His Leu
                85                  90                  95
Ile Leu Glu Gly Ile Trp Phe Lys Asp Gly Asn Arg Ala Ile Gln Ala
            100                 105                 110
Trp Lys Ser His Gly Pro Gly Leu Val Ala Ile Tyr Gly Ser Tyr Asn
        115                 120                 125
Arg Ile Thr Ala Cys Val Phe Asp Cys Phe Asp Glu Ala Asn Ser Ala
    130                 135                 140
Tyr Ile Thr Thr Ser Leu Thr Glu Asp Gly Lys Val Pro Gln His Cys
145                 150                 155                 160
Arg Ile Asp His Cys Ser Phe Thr Asp Lys Ile Thr Phe Asp Gln Val
                165                 170                 175
Ile Asn Leu Asn Asn Thr Ala Arg Ala Ile Lys Asp Gly Ser Val Gly
            180                 185                 190
Gly Pro Gly Met Tyr His Arg Val Asp His Cys Phe Phe Ser Asn Pro
        195                 200                 205
Gln Lys Pro Gly Asn Ala Gly Gly Gly Ile Arg Ile Gly Tyr Tyr Arg
    210                 215                 220
Asn Asp Ile Gly Arg Cys Leu Val Asp Ser Asn Leu Phe Met Arg Gln
225                 230                 235                 240
Asp Ser Glu Ala Glu Ile Ile Thr Ser Lys Ser Gln Glu Asn Val Tyr
                245                 250                 255
Tyr Gly Asn Thr Tyr Leu Asn Cys Gln Gly Thr Met Asn Phe Arg His
            260                 265                 270
Gly Asp His Gln Val Ala Ile Asn Asn Phe Tyr Ile Gly Asn Asp Gln
        275                 280                 285
Arg Phe Gly Tyr Gly Gly Met Phe Val Trp Gly Ser Arg His Val Ile
    290                 295                 300
```

```
Ala Cys Asn Tyr Phe Glu Leu Ser Glu Thr Ile Lys Ser Arg Gly Asn
305                 310                 315                 320
Ala Ala Leu Tyr Leu Asn Pro Gly Ala Met Ala Ser Glu His Ala Leu
                325                 330                 335
Ala Phe Asp Met Leu Ile Ala Asn Asn Ala Phe Ile Asn Val Asn Gly
            340                 345                 350
Tyr Ala Ile His Phe Asn Pro Leu Asp Glu Arg Arg Lys Glu Tyr Cys
        355                 360                 365
Ala Ala Asn Arg Leu Lys Phe Glu Thr Pro His Gln Leu Met Leu Lys
    370                 375                 380
Gly Asn Leu Phe Phe Lys Asp Lys Pro Tyr Val Tyr Pro Phe Phe Lys
385                 390                 395                 400
Asp Asp Tyr Phe Ile Ala Gly Lys Asn Ser Trp Thr Gly Asn Val Ala
                405                 410                 415
Leu Gly Val Glu Lys Gly Ile Pro Val Asn Ile Ser Ala Asn Arg Ser
            420                 425                 430
Ala Tyr Lys Pro Val Lys Ile Lys Asp Ile Gln Pro Ile Glu Gly Ile
        435                 440                 445
Ala Leu Asp Leu Asn Ala Leu Ile Ser Lys Gly Ile Thr Gly Lys Pro
    450                 455                 460
Leu Ser Trp Asp Glu Val Arg Pro Tyr Trp Leu Lys Glu Met Pro Gly
465                 470                 475                 480
Thr Tyr Ala Leu Thr Ala Arg Leu Ser Ala Asp Arg Ala Ala Lys Phe
                485                 490                 495
Lys Ala Val Ile Lys Arg Asn Lys Glu His Phe Ile Gly Arg Glu
            500                 505                 510
```

(2) INFORMATION FOR SEQ ID NO:5:
  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 29 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear
  (ii) MOLECULE TYPE: DNA (synthetic)
  (iii) HYPOTHETICAL: NO
  (iv) ANTI-SENSE: NO
  (ix) FEATURE:

(A) NAME/KEY: misc_feature
(B) LOCATION: 1..29
(D) OTHER INFORMATION: /note= "Nucleotide sequence encoding peptide AC-3."
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCNGGRAART ARTANCCDAT NGCRTCRTG 29

(2) INFORMATION FOR SEQ ID NO:6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (synthetic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..23
(D) OTHER INFORMATION: /note= "Nucleotide sequence encoding peptide AC-4."
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TAYATGGAYT TYAAYGTNGA RGG 23

(2) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (synthetic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8

```
        (D) OTHER INFORMATION: /note= "Nucleotides 3 through 8
              encode a BamHI site."
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGGATCCAT GCAGCAGACC GGTACTGCAG AA                              32

(2) INFORMATION FOR SEQ ID NO:8:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 30 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: DNA (synthetic)

   (iii) HYPOTHETICAL: NO
    (iv) ANTI-SENSE: NO
    (ix) FEATURE:
          (A) NAME/KEY: misc_feature
          (B) LOCATION: 4..9
          (D) OTHER INFORMATION: /note= "Nucleotides 4 through 9
              encode a BamHI site."
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGCGGATCCC CTAGATTACT ACCATCAAAA                                 30

(2) INFORMATION FOR SEQ ID NO:9:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 34 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: DNA (synthetic)
   (iii) HYPOTHETICAL: NO
    (iv) ANTI-SENSE: NO
    (ix) FEATURE:
          (A) NAME/KEY: misc_feature
          (B) LOCATION: 1..34
```

(D) OTHER INFORMATION: /note= "Nucleotide sequence derived from the CHB-2 peptide."
(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /note= "Nucleotides 3 through 8 encode a BamHI site."
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGGGATCCCA RATYGCCGAY GGNACNTATA AAGA 34

(2) INFORMATION FOR SEQ ID NO:10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (synthetic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..29
(D) OTHER INFORMATION: /note= "Nucleotide sequence derived from the CHB-3 peptide."
(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /note= "Nucleotides 3 through 8 encode a BamHI site."
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGGGATCCGG CNSKATTGCG TTCRTCAAA 29

(2) INFORMATION FOR SEQ ID NO:11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (synthetic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..34
(D) OTHER INFORMATION: /note= "Oligonucleotide used for amplification of the chondroitinase B coding sequence."
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGCGGATCCA TGCAGGTGTT GCTCAAATGA AACT 34

(2) INFORMATION FOR SEQ ID NO:12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (synthetic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGGAATCAAT TCACCGGG

We claim:

1. A purified chondroitinase degrading enzyme isolated from bacteria.

2. The enzyme of claim 1 selected from the group consisting of chondroitinase AC and chondroitinase B from *Flavobacterium heparinum*.

3. The enzyme of claim 2 expressed in bactera from a gene isolated from *Flavobacterium heparinum*.

4. The enzyme of claim 2 where the enzyme is chondroitinase AC and has a molecular weight between 72,000 and 82,000 Daltons and is capable of degrading chondroitin sulfate A and chondroitin sulfate C.

5. The enzyme of claim 2 where the enzyme is chondroitinase B and has a molecular weight between 52,700 and 57,300 Daltons and is capable of degrading dermatan sulfate or chondroitin sulfate B.

6. The enzyme of claim 4 encoded by the nucleotide sequence of Sequence ID No. 1 and sequences having conservative or degenerative substitutions thereof.

7. The enzyme of claim 4 having the amino acid sequence of Sequence ID No. 2 and sequences having conservative substitutions thereof.

8. The enzyme of claim 5 encoded by the nucleotide sequence of Sequence ID No. 3 and sequences having conservative or degenerative substitutions thereof.

9. The enzyme of claim 5 having the amino acid sequence of Sequence ID No. 4 and sequences having conservative substitutions thereof.

10. The enzyme of claim 1 further comprising a pharmaceutically acceptable carrier.

11. An isolated nucleotide sequence encoding an enzyme selected from the group consisting of chondroitinase AC and chondroitinase B from *Flavobacterium heparinum*.

12. The sequence of claim 11 naturally occurring in *Flavobacterium heparinum*.

13. The sequence of claim 11 where the enzyme is chondroitinase AC and has a molecular weight between 72,000 and 82,000 Daltons and is capable of degrading chondroitin sulfate A and chondroitin sulfate C.

14. The sequence of claim 11 where the enzyme is chondroitinase B and has a molecular weight between 52,700 and 57,300 Daltons and is capable of degrading dermatan sulfate or chondroitin sulfate B.

15. The sequence of claim 4 having the nucleotide sequence of Sequence ID No. 1 or sequences having conservative or degenerative substitutions thereof.

16. The sequence of claim 13 encoding the amino acid sequence of Sequence ID No. 2 or sequences having conservative substitutions thereof.

17. The sequence of claim 14 having the nucleotide sequence of Sequence ID No. 3 or sequences having conservative or degenerative substitutions thereof.

18. The sequence of claim 14 encoding the amino acid sequence of Sequence ID No. 4 or sequences having conservative substitutions thereof.

19. A method for purifying a chondroitin lyase from bacteria comprising:

lysing the bacteria;

extracting proteins from the periplasmic space of the lysed bacteria;

separating the extracted proteins by cation exchange chromatography using a salt or pH gradient;

separating the fractions having enzymatic activity obtained by elution of the cation exchange chromatography matrix by chromatography on a sulfated cellulose resin using a salt or pH gradient;

separating the fractions having enzymatic activity obtained by elution of the sulfated cellulose resin on hydroxyapatite using a salt or pH gradient;

separating the fractions having enzymatic activity obtained by elution of the hydroxyapatite by chromatography using cation exchange chromatography using a salt or pH gradient; and

separating the fractions with enzymatic activity on the basis of molecular weight.

FIG. 1

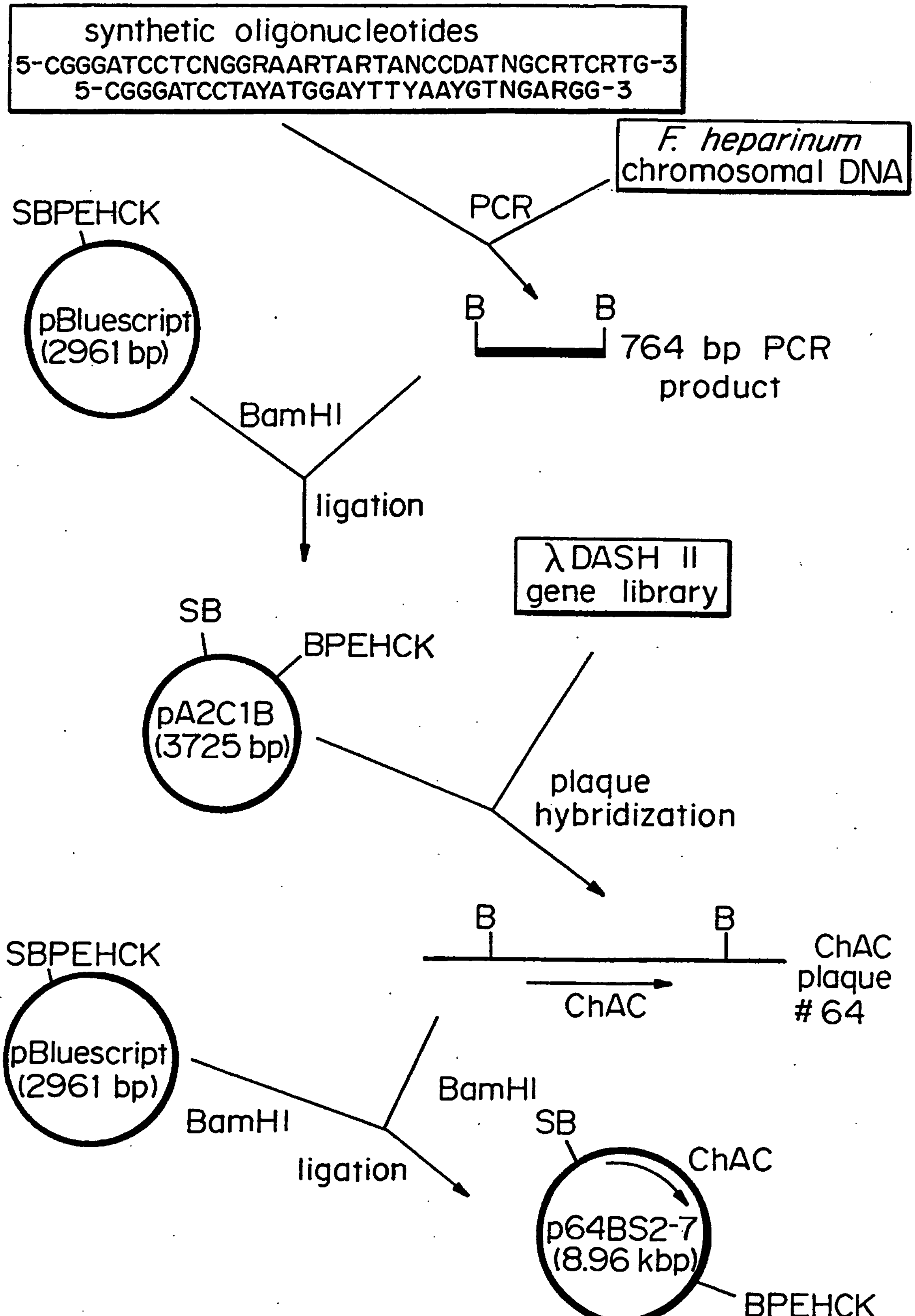
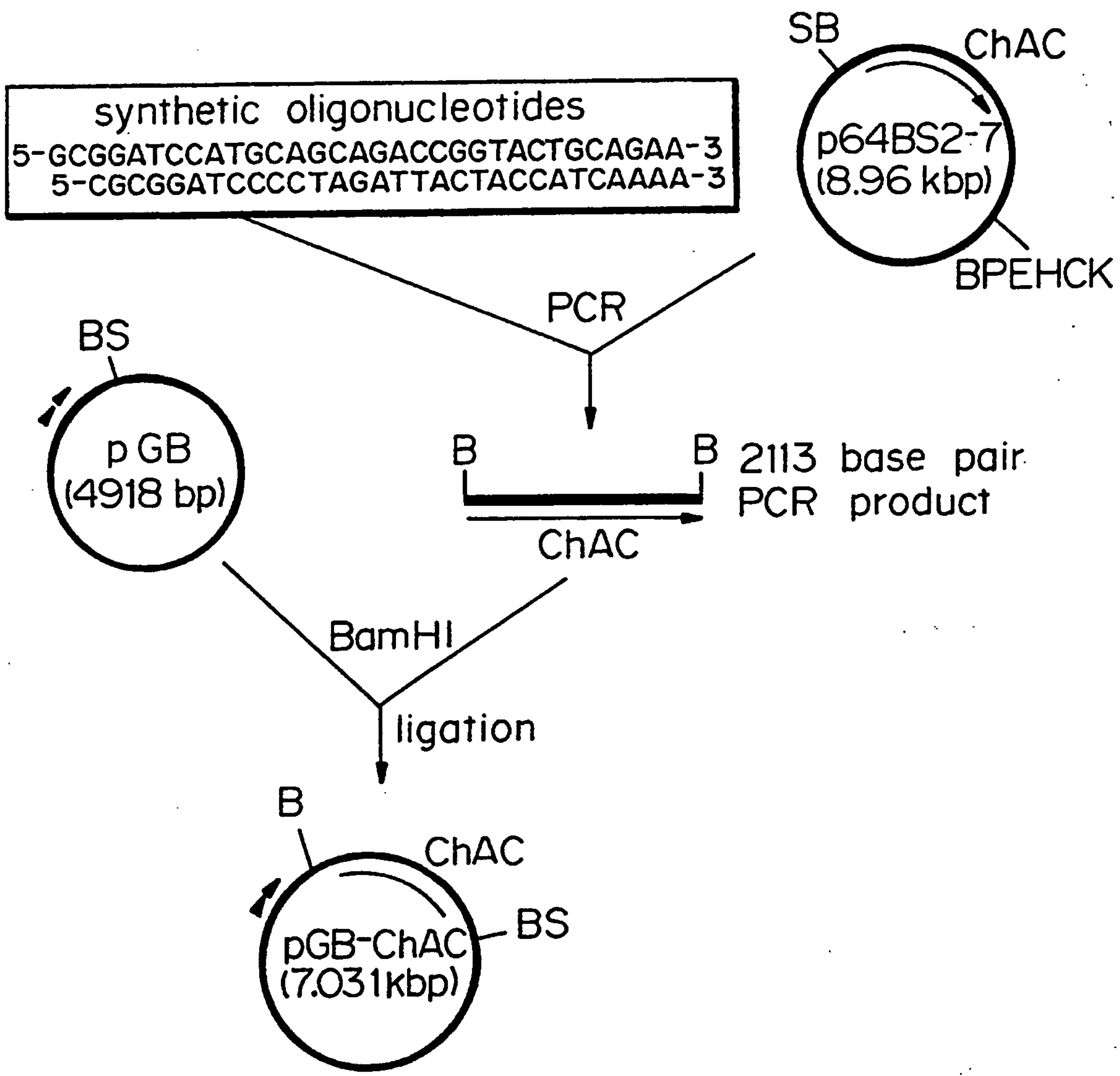

synthetic oligonucleotides
5-CGGGATCCTCNGGRAARTARTANCCDATNGCRTCRTG-3
5-CGGGATCCTAYATGGAYTTYAAYGTNGARGG-3
F. heparinum
chromosomal DNA
PCR
SBPEHCK
pBluescript
(2961 bp)
B
B
764 bp PCR
product
BamHI
ligation
λ DASH II
gene library
SB
BPEHCK
pA2C1B
(3725 bp)
plaque
hybridization
B
B
ChAC
plaque
64
ChAC
SBPEHCK
pBluescript
(2961 bp)
BamHI
BamHI
SB
ligation
ChAC
p64BS2-7
(8.96 kbp)
BPEHCK

FIG. 2


synthetic oligonucleotides
5-GCGGATCCATGCAGCAGACCGGTACTGCAGAA-3
5-CGCGGATCCCCTAGATTACTACCATCAAAA-3
SB
ChAC
p64BS2-7
(8.96 kbp)
BPEHCK
PCR
BS
pGB
(4918 bp)
B
B
2113 base pair
PCR product
ChAC
BamHI
ligation
B
ChAC
BS
pGB-ChAC
(7.031kbp)

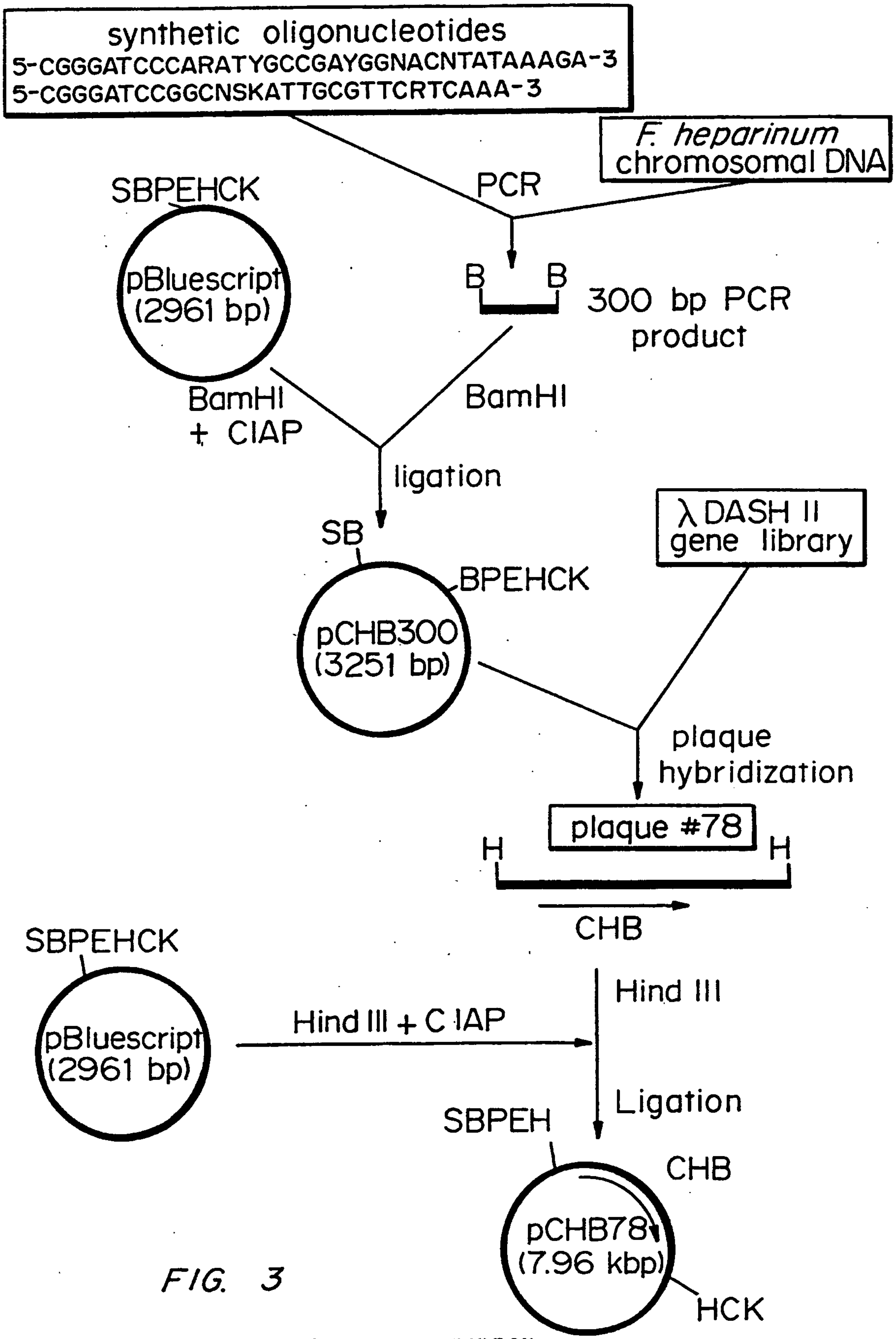
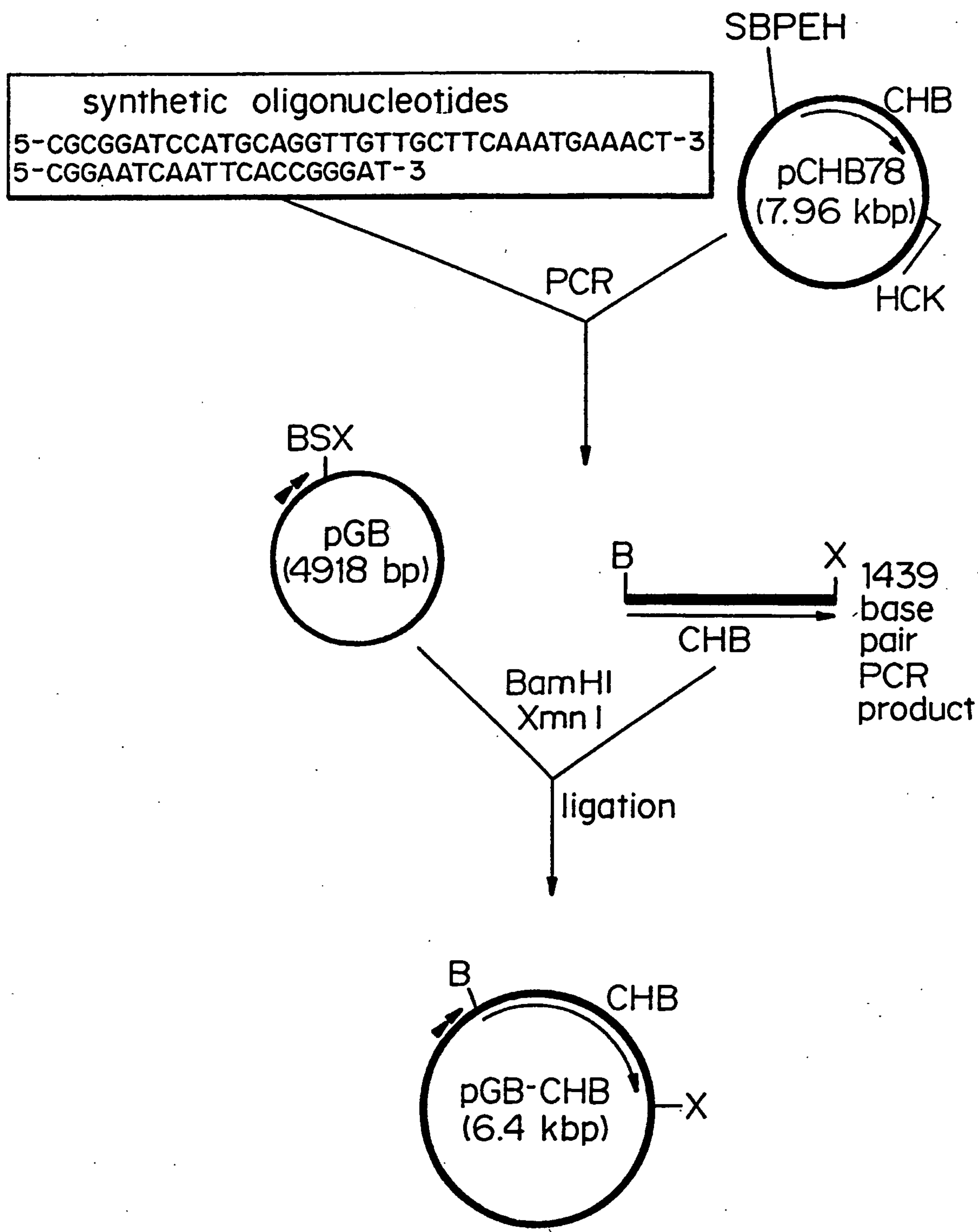

synthetic oligonucleotides
5-CGGGATCCCARATYGCCGAYGGNACNTATAAAGA-3
5-CGGGATCCGGCNSKATTGCGTTCRTCAAA-3
F. heparinum chromosomal DNA
PCR
SBPEHCK
pBluescript (2961 bp)
B B
300 bp PCR product
BamHI + CIAP
BamHI
ligation
SB
BPEHCK
pCHB300 (3251 bp)
λ DASH II gene library
plaque hybridization
plaque #78
H H
CHB
SBPEHCK
pBluescript (2961 bp)
Hind III + C IAP
Hind III
Ligation
SBPEH
CHB
pCHB78 (7.96 kbp)
HCK

FIG. 3

FIG. 4


SBPEH
synthetic oligonucleotides
5-CGCGGATCCATGCAGGTTGTTGCTTCAAATGAAACT-3
5-CGGAATCAATTCACCGGGAT-3
CHB
pCHB78
(7.96 kbp)
HCK
PCR
BSX
pGB
(4918 bp)
B
X
CHB
1439 base pair PCR product
BamHI
Xmn I
ligation
B
CHB
pGB-CHB
(6.4 kbp)
X

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/08560

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/88 C12N15/60 //(C12N9/88,C12R1:20)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCHEM. J. (1975), 151(1), 121-9 , MICHELACCI, YARA M. ET AL 'Comparative study between a chondroitinase B and a chondroitinase AC from Flavobacterium heparinium. Isolation of a chondroitinase AC-susceptible dodecasaccharide from chondroitin sulfate B'	1,2
Y	see the whole document	3-19

Y	DATABASE WPI Section Ch, Week 9419 Derwent Publications Ltd., London, GB; Class D16, AN 94-155922 & JP,A,06 098 769 (TAIYO FISHERY CO LTD) , 12 April 1994 see abstract	3-18

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
2 November 1995	29. 11. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gurdjian, D

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE WPI Section Ch, Week 8046 Derwent Publications Ltd., London, GB; Class B04, AN 80-81758C & JP,A,55 127 988 (SEIKAGAKU KK) , 4 October 1980 see abstract ---	19
Y	US,A,4 390 628 (JOHANSEN JACK T) 28 June 1983 see abstract ---	19
X	BIOCHIM. BIOPHYS. ACTA (1987), 923(2), 291-301 , MICHELACCI, YARA M. ET AL 'Isolation and characterization of an induced chondroitinase ABC from Flavobacterium heparinum' see the whole document -----	1,2

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intional Application No
PCT/US 95/08560

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-4390628	28-06-83	AT-T-	6076	15-02-84
		EP-A,B	0019477	26-11-80
		SU-A-	1184434	07-10-85
		US-A-	4388406	14-06-83
		AT-T-	5976	15-02-84
		EP-A,B	0019474	26-11-80
		JP-C-	1586049	31-10-90
		JP-B-	2012556	20-03-90
		JP-A-	56035983	08-04-81
		JP-B-	1033158	12-07-89
		JP-C-	1549173	09-03-90
		JP-A-	56035984	08-04-81
		US-A-	4340675	20-07-82